MULTIPLE STRESS-INDUCIBLE PEROXIDASE PROMOTER DERIVED FROM *IPOMOEA BATATAS*

FIELD OF THE INVENTION

The present invention relates to a multiple stress inducible promoter, more particularly to a multiple stress inducible peroxidase promoter derived from a sweetpotato (*Ipomoea batatas*), an expression vector for production of transgenic plants with enhanced tolerance to multiple stress containing the promoter, a transgenic plant cell line and plants transfected by the expression vector, and a method for generating the transgenic plants.

BACKGROUND

When plants get stress including environmental or biological stress from pathogenic bacteria, noxious insects, or viruses, oxygen inside which is an essential ingredient for a life changes into reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, etc, causing serious disorders. In order to eliminate such active

oxygen, a living body has macromolecular anti-oxidant enzymes such as superoxide dismutase (SOD: EC 1.15.1.1), peroxidase (POD) and catalase (CAT), and low molecular weight anti-oxidant substances such as vitamin C, vitamin E, glutathion, etc.

Peroxidase is an enzyme reducing hydrogen peroxide in the presence of electron donors, and is largely found in plant cells. Owing to its high sensitivity to enzyme reaction, peroxidase has been used as a reagent for many clinical tests. In addition to the importance in industry, peroxidase also draws an attention of scientists since it plays an important role in plant reaction against stress from outside. In general, the activity of plant peroxidase increases by various environmental stresses. In particular, plant culture cells show high activity of peroxidase because the cells are cultured under huge oxidative stress. According to an earlier report, peroxidase is mass-produced in sweetpotato culture cells more than in any other plant culture cells (*Phytochemistry*, 39, 981-984, 1995).

As of today, genes coding peroxidase included

in some particular plants have been found in about 20 different plant species such as horseradish, barley, wheat, rape, *Arabidopsis thaliana*, tobacco, spinach, rice plant, etc. Recently, a total base sequence of Araidopsis has been identified, from which 73 peroxidase genes have been confirmed (*Gene*, 288, 129-138, 2002). But, the function of each individual peroxidase has not been explained yet. Peroxidase genes of a sweetpotato have been first reported by the present inventors. Particularly, the present inventors have separated three acidic peroxidase genes (*swpa1*, *swpa2*, *swpa3*) and a neutral peroxidase gene (*swpn1*) from sweetpotato culture cells, and have reported that those genes are expressed specifically in sweetpotato culture cells and found multiply in genome, and have further confirmed that peroxidase can be mass-produced stably by transfecting cells or plants with either some parts or a whole peroxidase gene (*Mol. Gen. Genet.*, 255, 382-391, 1997; *Mol. Genet. Genet.*, 261, 941-947, 1999).

In pervious studies, the present inventors separated novel acidic peroxidase genes '*swpa4*, '*swpa5*, and '*swpa6*' along with basic peroxidase

genes '*swpb1*', '*swpb2*' and '*swpb3*' whose base sequences were all disclosed (Korea Patent Application #2003-28811; *Mol. Genet. Genomics*, 261, 941-947, 2003). *Swpa4* was expressed strongly in sweetpotato culture cells but was not expressed in normal plant tissues. *Swpa4* was highly expressed not only by biological stress like pathogenic bacteria (*Pectobacterium chrysanhemi*, KCTC 2569) but also by non-biological stresses such as wounding, methyl viologen and hydrogen peroxide having a herbicial activity by generating active oxygen, NaCl, methyl jasmonate, abscisic acd, low temperature of 15℃ and high temperature of 37℃, etc.

Thus, the present inventors have separated genomic DNA from a sweetpotato which is coding peroxidase expressed actively not only by biological stress but also by many other physical or chemical stresses, and completed this invention by confirming that a promoter of the same is valuable enough in industry.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a multiple stress-resistant promoter sequence including a base sequence represented by SEQ. ID. No 2.

It is also an object of this invention to provide an expression vector for the production of a multiple stress-resistant transformant including the above promoter sequence, target substance coding sequence and transcription terminator sequence.

It is a further object of this invention to provide multiple stress-resistant transgenic cells prepared by transfecting host plant cells with the above expression vector.

It is also an object of this invention to provide a multiple stress-resistant transgenic plant prepared by transfecting a host plant with the above expression vector using an *Agrobacterium*.

It is also an object of this invention to provide a preparation method for the above multiple stress-resistant transgenic plant.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

In order to achieve the above object, the present invention provides a multiple stress-resistant promoter sequence including a base sequence represented by SEQ. ID. No 2.

The present invention also provides an expression vector for the production of a multiple stress-resistant transformant including the above promoter sequence, target substance coding sequence and transcription terminator sequence.

The present invention further provides multiple stress-resistant transgenic cells prepared by transfecting host plant cells with the above expression vector.

The present invention also provides a multiple-stress-resistant transgenic plant prepared by transfecting a host plant with the above expression vector using an *Agrobacterium*.

The present invention also provides a preparation method for the above multiple stress-resistant transgenic plant.

"*SWPA4* promoter" is a base sequence located on -1 ~ -2433 region of a promoter sequence having

a base sequence represented by SEQ. ID. No 11, and induces transcription of a related gene under a required condition.

"Active fragment of *SWPA4* promoter" is a base sequence containing some of base sequence located on -1 ~ -2433 region of swpa4 genomic gene sequence represented by SEQ. ID. No 1, and endows a gene properly linked with a *SWPA4* promoter activity.

"Transformant" means a plant culture cell line or a plant transfected with DNA construct composed of *SWPA4* promoter and properly linked DNA sequence coding relevant substances.

"Multiple stress" includes biological or non-biological stress, for example, wound, active oxygen species, heat, moisture, temperature, salt, air pollution, UV, heavy metals, chemical herbicides, pathogenic bacteria, etc.

Hereinafter, the present invention is described in detail.

The present invention provides a multiple stress-resistant promoter sequence including a base sequence represented by SEQ. ID. No 2.

The promoter sequence of the present

invention is preferably selected from a group consisting of base sequences represented by SEQ. ID. No 2 ~ No 11. The promoter sequence represented by SEQ. ID. No 11 is a whole promoter sequence located in front of transcription beginning region (ATG) included in peroxidase *SWPA4* genomic gene derived from a sweetpotato which is represented by SEQ. ID. No 1. SEQ. ID. No 2, SEQ. ID. No 3, SEQ. ID. No 4, SEQ. ID. No 5, SEQ. ID. No 6, SEQ. ID. No 7, SEQ. ID. No 8, SEQ. ID. No 9 and SEQ. ID. No 10 are all fragment sequences located on the -110^{th}, -177^{th}, -306^{th}, -366^{th}, -433^{rd}, -818^{th}, -1199^{th}, -1467^{th} and -1934^{th} sites each from the end of a whole *SWPA4* promoter sequence (located just in front of transcription beginning region).

A full-length *SWPA4* promoter having a base sequence represented by SEQ. ID. No 11 includes specific regions for regulatory elements of various eukaryotic promoters, and TATA box (TATTTAA) for transcription beginning locates on -92^{nd} ~ -86^{th} site. RSTGACTMANA, a consensus sequence of AP-1 which is known as an attachment site for a transcription regulating protein and a major element reacting against active oxygen

species, locates on between -431st and -421st (Lucibello, FC. *et al.*, *Oncogene*, 8, 1667-1672, 1993). A consensus sequence of ELRE, TTGACC (Rushton, PJ. *et al.*, *EMBO J*, 15, 5690-5700, 1996), whose expression is strongly induced by elicitor generated by a defense mechanism of plant against pathogenic bacteria infection or wound, locates on regions between -2227 and -2232 and between -1329 and -1334 as an inverted repeat sequence. TAACGTA, a consensus sequence of GARE whose expression is regulated by a plant hormone 'gibberellin' (GA), locates on the region between -382 and -376 (Sutoh, K. *et al.*, *Plant J*, 34, 636-645, 2003). AWTTCAAA, a consensus sequence of ERE whose expression is regulated by a plant hormone 'ethylene' which is related to ripening and aging of a fruit, locates on the region between -192 and -185 (Itzhaki, H. *et al.*, *Proc Natl Acad Sci USA*, 91, 8925-8929, 1994). W-box, on which WRKY protein which plays an important role in resistance against a disease after being expressed by salicylic acid (SA) is attached, locates on the regions between -1993 ~ -1989, and between -1032 ~ -1028 with repeat of TTGAC and also between -2227 ~ -2231 and between -1329 ~ -1333 as an inverted repeat sequence this

time (Yu, D. *et al.*, *Plant Cell*, 13, 1527-1540, 2001). AGAAN, a consensus sequence which is a heat shock element (HSE), locates on the promoter region between -182 and -178 (Fernandes, M. *et al.*, *Nucleic Acids Res*, 22, 167-173, 1994) (see FIG. 1a).

In the preferred embodiment of the invention, the present inventors prepared promoter sequences represented by SEQ. ID. No 2 ~ No 11 from peroxidase SWPA4 genomic gene represented by SEQ. ID. No 1 included in a sweetpotato, and an expression vector containing the same. Transgenic tobacco cells including various size promoters were also produced by transfecting tobacco culture cells with the expression vector prepared above. An activity of the promoter was investigated by using protoplasts of tobacco cells. As a result, deleted promoter fragments having different sizes, which were represented by SEQ. ID. No 2 ~ No 11, showed similar activity to or over 4.5-fold (but less than 8.5-fold) higher promoter activity than CaMV35S promoter of a control group (see FIG. 3a and 3b). A transgenic tobacco plant was produced by inserting an expression vector containing

various size deleted promoter fragments into a tobacco leaf section using *Agrobacterium*. Then, stress was induced therein. Promoter activity was investigated after inducing stress. As a result, the activity of GUS (a target gene included in an expression vector) was increased after the treatment of pathogenic bacteria, methyl viologen or causing wound, at least twice as much as before the treatment (see Table 1). Therefore, promoter sequences of the present invention represented by SEQ. ID. No 2 ~ No 11 were proved to have higher promoter activity than any conventional promoters, and the activity was strongly enhanced by stress. So, promoter sequences of the present invention can be effectively used for the development of an environmental stress-resistant plant and the production of valuable substances by taking an advantage of the transgenic plant cells obtained thereby.

The present invention also provides an expression vector for the production of a multiple stress-resistant transformant including the above promoter sequence, target substance coding sequence and transcription terminator sequence.

The promoter sequence included in the expression vector of the present invention is preferably selected from base sequences represented by SEQ. ID. No 2 ~ No 11.

It is also preferred for a target substance of the invention to include various proteins or peptides having pharmaceutical effects or any other substance giving resistance against stress to a transformant. In the preferred embodiment of the present invention, expression vectors having deletion promoters in various sizes were constructed by cloning each sequence of the promoter represented by SEQ. ID. No 2 ~ No 11 into plasmid vector pBI1221 (CaMV35S promoter, GUS coding sequence and NOS transcription terminator sequence were included) provided by Clontech, Co. Each expression vector prepared above was named, according to the length of a promoter sequence, 'p2433', 'p1934', 'p1467', 'p1199', 'p818', 'p433', 'p366', 'p306', 'p177' and 'p110'. GUS was used as a target gene for the expression vector of the invention. But, GUS could be replaced by any other target valuable substance coding sequence to construct an expression vector producing a target valuable substance keeping resistance against

stress.

The present invention further provides multiple stress-resistant transgenic cells prepared by transfecting host plant cells with the above expression vector and a multiple stress-resistant transgenic plant prepared by transfecting a host plant with the above expression vector using an *Agrobacterium*.

For the preparation of transgenic cells and a transgenic plant of the present invention, the expression vector preferably contained a promoter sequence selected from a group consisting of sequences represented by SEQ. ID. No 2 ~ No 11. For producing transgenic cells of the present invention, a host cell was preferably selected from a group consisting of tobacco, major agricultural crops such as rice, sweetpotato, etc, and medicinal plants including ginseng. For producing a transgenic plant of the present invention, a host plant was preferably selected from a group consisting of tobacco, major crops such as rice, sweetpotato, etc, and medicinal plants including ginseng.

In the preferred embodiment of the present

invention, cells of a tobacco, *Nicotiana tabacum*, were transfected with expression vectors p110, p177, p306, p366, p433, p818, p1199, p1467, p1934 and p2433, each including a promoter sequence selected from a group consisting of sequences represented by SEQ. ID. No 2 ~ No 11, using an *Agrobacterium*, resulting in the preparation of transgenic tobacco cells expressing the above expression vectors respectively. Among those transgenic cells, the one cell line that was transfected with expression vector p1467 showed the highest promoter activity, so that it was named 'p1467 (*Nicotiana tabacum cv. Xanthi*) cell line' and deposited at KCTC (Korean Collection for Type Culture) of KRIBB (Korea Research Institute of Bioscience and Biotechnology) on February 10, 2004 (Accession No: KCTC 10594BP).

The present invention also provides a preparation method of a multiple stress-resistant transgenic plant comprising the following steps:

1) Constructing an expression vector containing each of a promoter sequence selected from a group consisting of base sequences represented by SEQ. ID. No 2 ~ No 11, a target

valuable substance coding sequence and a transcription terminator sequence; and

2) Transfecting a host plant with the expression vector of the above step 1) using an *Agrobacterium*.

BRIEF DESCRIPTION OF THE DRAWINGS

The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

FIG. 1a and FIG. 1b (continuation of FIG. 1a) show a base sequence of genomic gene *SWPA4* coding peroxidase of the present invention originated from a sweetpotato and an amino acid sequence translated from the same. The part of base sequence marked with (-) is a promoter sequence (FIG. 1a), the parts marked with base sequence and amino acid sequence together are exons and the parts marked with only base sequence are introns (FIG. 1b).

FIG. 2 is a schematic diagram showing the structures of genomic gene *SWPA4* coding peroxidase

of the present invention and an expression vector for the production of deletion mutants that include deletion promoters. E1, E2 and E3 are exons, and I1 and I2 are introns.

FIG. 3a is a graph showing the results of an investigation of a promoter activity using deletion mutants deficient in different promoter regions (p2433, p1934, p1467, p1199, p818, p433).

FIG. 3b is a graph showing the results of an investigation of a promoter activity using deletion mutants deficient in different promoter regions (p433, p366, p306, p177, p110). The promoter activity of CsMV 35S was measured as a control in FIG. 3a and FIG. 3b.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and

improvements within the spirit and scope of the present invention.

Example 1: Separation of sweetpotato-originated peroxidase genomic DNA *SWPA4* and analysis on base sequence of the same

Genomic DNA of sweetpotato-originated peroxidase gene *SWPA4* was separated by using GenomWalker kit (Clontech) following the manufacturer's instruction. 2.5 μg of a sweetpotato genomic DNA, extracted by the general method, was digested with restriction enzymes *EcoR*V, *Dra*I, *Pvu*II, *Ssp*I, etc. The digested genomic DNA was purified using phenol/chloroform/ethanol. GenomWalker library was constructed by linking the purified genomic DNA and an adaptor supplied by the kit by using ligase. Based on the library, *SWPA4* genomic DNA was obtained by PCR. PCR was performed with GSP1 primer represented by SEQ. ID. No 12, which was established based on the information on 5'-end base sequence of *SWPA4* cDNA, and an adaptor primer AP1 represented by SEQ. ID. No 13, which was

supplied by the kit above, by using the above genomic DNA as a template. PCR reaction was induced at 94℃ for 25 seconds and at 72℃ for 4 minutes, which was repeated 7 times, and further at 94℃ for 25 seconds and 67℃ for 4 minutes, which was repeated 32 times. After PCR, electrophoresis was performed to confirm some of the products. After diluting the primary PCR product by 50 times, PCR was performed again with GSP2 primer represented by SEQ. ID. No 14, which was prepared on the basis of the information on the base sequence of 5'-end of a sweetpotato originated peroxidase *SWPA4* cDNA (Korea Patent Application #2003-28811), and an adaptor primer AP2 represented by SEQ. ID. No 15, which was supplied by the kit. The PCR condition was as follow; at 94℃ for 25 seconds and at 72℃ for 4 minutes (5 cycles), at 94℃ for 25 seconds and 67℃ for 4 minutes (22 cycles). Then, electrophoresis was performed to confirm PCR product. The PCR product was cloned into pGEM-T Easy vector (Promega) and base sequence was investigated. Exon region of *SWPA4* gene was amplified by using a primer represented by SEQ. ID. No 16 and an adaptor primer AP1 represented by SEQ.

ID. No 13, likewise, intron region of *SWPA4* gene was amplified by using a primer represented by SEQ. ID. No 17 and an adaptor primer AP2 represented by SEQ. ID. No 15.

The amplified PCR product was cloned into pGEM-T Easy vector following the same method above, and a base sequence was determined, so that sequence of the genomic gene represented by SEQ. ID. No 1 was obtained (FIG. 1a and 1b), and named '*SWPA4*'. *SWPA4* separated from a sweetpotato genome was 3945 bp in total length and composed of three exons, two introns and a 2433 bp long promoter. Base sequence of exon of the genomic gene was confirmed to be identical with that of *SWPA4* cDNA (Korea Patent Application #2003-28811), and 5' of each intron began with GT and 3' of them ended with AG, suggesting that it was keeping the rule of GT-AG (FIG. 1a, 1b and FIG. 2).

Example 2: Investigation of a promoter activity of peroxidase genomic DNA *SWPA4*

A promoter of wild type *SWPA4* was composed of base sequence represented by SEQ. ID. No 11, ranging from upstream of translation starting

point of peroxidase *SWPA4* genomic DNA to -2433 bp point (FIG. 1a and 1b). The characteristics of the base sequence of *SWPA4* promoter was investigated by using PLACE and Transfac provided by Computational Biology & Informatics Laboratory.

As a result, *SWPA4* promoter was confirmed to have regulatory elements of various eukaryotic promoters, and TATA-box for the translation starting was located between -92 ~ -86 (Zhu, Q. *et al.*, *Plant Cell*, 14, 795-803, 2002). RSTGACTMANA (Lucibello, FC. *et al.*, *Oncogene*, 8, 1667-1672, 1993), a consensus sequence of AP1, which has been known as a relevant factor responding to active oxygens and a transcription regulatory protein is attached to, was located between -431 ~ -421. TTGACC (Rushton, PJ. *et al.*, *EMBO J*, 15, 5690-5700, 1996), a consensus sequence of ELRE which strongly induces a gene expression by elicitor generated by a defense mechanism against germ infection or wound in plants, was found between -2227 ~ -2232, and between -1329 ~ -1334 as an inverted repeat sequence. TAACGTA (Sutoh, K. *et al.*, *Plant J*, 34, 636-645, 2003), a consensus sequence of GARE whose expression is regulated by gibberellin (GA), a plant hormone in a plant, was located between -382

~ -376. AWTTCAAA (Itzhaki, H. *et al.*, *Proc Natl Acad Sci USA*, 91, 8925-8929, 1994), a consensus sequence of ERE whose expression is regulated by ethylene, a plant hormone related to ripening and aging of a fruit of a plant, was located between -192 ~ -185. W-box (Yu, D. *et al.*, *Plant Cell*, 13, 1527-1540, 2001), to which WRKY protein expressed by salicylic acid playing an important role in resistance against diseases is attached, was located as a repeat sequence of TTGAC at two regions between -1993 and -1989 and between -1032 and -1028, and located as an inverted repeat sequence at two other regions between -2227 and -2231 and between -1329 and -1333. AGAAN (Fernandes, M. *et al.*, *Nucleic Acids Res*, 22, 167-173, 1994), a consensus sequence of HSE (heat shock element) responding to heat shock was located between -182 and -178 of the promoter (FIG. 1a).

As explained hereinbefore, *SWPA4* promoter of the present invention includes many important factors recognizing various types of stress including ROS, so that it can be effectively used for the development of a stress-resistant plant standing against environmental stress.

Example 3: Preparation of deletion mutants of *SWPA4* promoter

<3-1> Preparation of deletion mutants of 2433 bp ~ 433 bp long promoters

In order to prepare deletion mutants of *SWPA4* promoter of the present invention, *SWPA4* promoter region was amplified by PCR using ExTaq polymerase (Takara) and sequence specific primers. At that time, primers represented by SEQ. ID. No 18 and No 24 were used to amplify a 2433 bp long promoter, primers represented by SEQ. ID. No 19 and No 24 were used to amplify a 1934 bp long promoter, primers represented by SEQ. ID. No 20 and No 24 were used to amplify a 1467 bp long promoter, primers represented by SEQ. ID. No 21 and No 24 were used to amplify a 1199 bp long promoter, primers represented by SEQ. ID. No 22 and No 24 were used to amplify a 818 bp long promoter, and primers represented by SEQ. ID. No 23 and No 24 were used to amplify a 433 bp long promoter. All the upstream primers (SEQ. ID. No 18 through No 23) were made to include a *Hind*III restriction

enzyme region and the downstream primers (SEQ. ID. No 24) were made to include a *Xba*I restriction enzyme region (FIG. 2).

After digesting the PCR product with restriction enzymes *Hind*III/*Xba*I, DNA fragments were sub-cloned into pBI221 plasmid vector (Clontech, CaMV35S promoter, GUS coding region and NOS terminator were included) which was already digested with the same restriction enzymes. At last, deletion mutation plasmid vectors in various lengths, each having different deletion constructions of *SWPA4* promoter (-2433, -1934, -1467, -1199, -818, and -433), were prepared and named 'p2433', 'p1934', 'p1467', 'p1199', 'p818' and 'p433'.

<3-2> Preparation of deletion mutants of the promoter less than 433 bp

In order to prepare deletion mutants having a promoter less than 433 bp, -433 bp long DNA fragment was amplified by PCR using ExTaq polymerase (Takara) and sequence specific primers, resulting in deletion promoter fragments in each 433, 366, 306, 177 and 110 bp length. At that

time, primers represented by SEQ. ID. No 25 and No 24 were used to amplify a 366 bp long promoter, primers represented by SEQ. ID. No 26 and No 24 were used to amplify a 177 bp long promoter, and primers represented by SEQ. ID. No 28 and No 24 were used to amplify a 110 bp long promoter. All the upstream primers (SEQ. ID. No 25 through No 28) were designed to have a *Pst*I restriction enzyme region.

Example 4: Investigation of a *SWPA4* promoter activity using tobacco protoplasts (transient assay)

By taking advantage of deletion mutants of *SWPA4* promoter, the promoter activity, according to the length of the deletion promoter, was investigated. First, a tobacco culture cell line BY-2 (*Nicotiana tabacum* L. cv. Bright yellow 2) was sub-cultured. Three days after the culture began, the cells were centrifuged to obtain cell membranes only, which were then treated with enzyme solution (30 mℓ; 1% cellulase R-10, 0.25% marcerozyme R-10, 60 mg MES, 30 mg BSA, 400 mM

mannitol, 1 mM $CaCl_2$, distilled water 28 mℓ) for 3 hours. Protoplasts were separated by centrifugation. The separated protoplasts were washed twice with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM MES-KOH, pH 5.0), and final cell concentration was adjusted to 2×10^6 cells/mℓ in MaMg solution (0.4 M mannitol, 0.1% MES, 15 mM $MgCl_2$, pH 5.6). 5 ㎍ of luciferase expression vector (Luc coding region was inserted in pBI221 for an internal control, Clontech), 300 ㎕ of protoplast solution and 300 ㎕ of PEG solution (40% PEG 3350, 100 mM $Ca(NO_3)_2$, 400 mM mannitol) were all mixed with 10 ㎍ of each deletion mutant plasmid vector DNA prepared in the <Example 4>, which were left at room temperature for 30 minuets. The mixture was washed with W5 solution by centrifugation, and suspended in 300 ㎕ of W5 solution, followed by further culture at 25℃ for 16 hours. Upon completing the culture, cells were collected to investigate the activities of luciferase and GUS. In particular, the activity of luciferase was investigated by using luciferase assay kit supplied by Promega, and the activity of GUS promoter in protoplasts where a deletion mutant plasmid vector was inserted was

investigated by measuring the amount of produced GUS protein according to the method of Jefferson *et al.* examining fluorescence by using MUG as a substrate (Jefferson *et al.*, *Plant Mol. Biol.*, 5, 387-405, 1987). The plasmid insertion efficiency into protoplast was also investigated by measuring the activity of luciferase inserted thereto at the same time.

As a result, -2433 *SWPA4* promoter showed about 5.3 fold higher activity than CaMV35S promoter. -1934 and -1467 promoters showed about 8.5 times higher activities and -1199 promoter showed about 7.5 times, -818 promoter showed about 5.1 times, and -433 promoter showed 4.8 times higher activity than a control (FIG. 3a). Based on that result, it was assumed that a transcription factor binding region suppressing expression was located between -2433 and -1934, and a transcription factor binding region promoting expression was located between -1467 showing the highest activity and -818 which was a turning point that the activity turned to decrease. -433 promoter having higher activity than CaMV35S promoter was re-made to be a shorter deletion structure, whose activity was then investigated.

As a result, the activity of the shorter deletion mutant decreased and reached similar level to that of CaMV35S promoter at -177. Therefore, a minimum promoter length that can keep higher *SWPA4* activity than CaMV35S was 301 bp and a binding region of a transcription regulation factor inducing a strong *SWPA4* activity was believed to be located between -306 and -177 (FIG. 3b).

Example 5: Analysis on the expression of GUS gene in a transgenic plant by using *SWPA4* promoter

<5-1> Preparation of a transgenic plant

A tobacco plant (*Nicotiana tabacum* cv. Xanthi) was used as a material for the preparation of a transgenic plant. Only the promoter regions of plasmid vectors p2433, p1467 and p433 including SWPA4 promoter deletion mutant were digested with *Hind*III and *Xba*I, which were inserted in pBI121 (Clontech), which was obtained in advance by being digested with the same restriction enzymes, resulting in a plant expression vector. CaMV35S promoter was inserted for a control. Each vector prepared above was inserted in *Agrobacteria* by

using *Agrobacterium tumefaciens* LBA4404 (ATCC), after which tobacco leaf fragments were infected with the same. The infected leaf fragments were cultured in MS medium containing 200 mg/ℓ kanamycin and 300 mg/ℓ claforan (Murashige T and Skoog F, *Physiol Plant*, 15, 473-497, 1962). Then, a transgenic plant was selected, which was acclimated by rooting and shooting. The plant was transplanted in a small flowerpot and became ready to be used as a test material.

In order to confirm if SWPA4 promoter deletion mutant was correctly inserted in a transgenic plant, PCR was performed with a pair of NPTII primer represented by SEQ. ID. No 29 and No 30 and a pair of primer for the amplification of 433 bp promoter represented by SEQ. ID. No 23 and No 24. When a pair of NPTII primer was used, PCR was performed at 95℃ for 1 minute, at 65℃ for 1 minute and at 72℃ for 1 minute (30 cycles). When a pair of primer for the amplification of 433 bp promoter was used, PCR was performed at 95℃ for 1 minute, at 62℃ for 1 minute and at 72℃ for 1 minute (30 cycles). Electrophoresis on agarose followed PCR to confirm PCR product.

As a result, 0.7 kb DNA fragment amplified by

the pair of NPTII primer and 433 bp DNA fragment amplified by the pair of primer for the amplification of 433 bp promoter were detected in a transgenic plant. Thus, it was confirmed that a foreign gene was successfully inserted in the transgenic plant.

<5-2> Investigation of GUS expression by stress in a transgenic plant

In order to investigate the expression of SWPA4 promoter by an environmental stress in a transgenic plant, the transgenic plant was treated with methyl viologen (referred as 'MV' hereinafter) and pathogenic bacteria, and was wounded as well to measure the activity of GUS induced thereby.

At first, the expression of *SWPA4* promoter by a wound was investigated. Particularly, a transgenic plant was wounded and then the activity of GUS induced thereby was measured. At that time, regarding GUS activity in a CaMV35S promoter plant as 100%, GUS activity in a *SWPA4* promoter transgenic plant was represented by a relative activity. As a result, when a transgenic plant in

which pBI121 vector containing CaMV35S promoter-GUS gene was inserted was treated with nothing (meaning 'control'), the GUS activity (pmol/minute/mg protein) was 7,200 ±135, which was hardly changed by stress like wound. In the case of transgenic plants each inserted with p2433, p1467 and p433 vector, the expression of GUS increased greatly three days after wounding. The expression of GUS in a transgenic plant infected with p1467 vector was 2.5 fold higher by a wound than that in CaMV35 promoter. The expression of GUS by a wound in a transgenic plant infected with p2433 vector could not compete with that in CaMV35S promoter, but the expression of GUS in a transgenic plant infected with p433 was as much as that in CaMV 35S promoter (Table 1). Therefore, it was confirmed that a promoter activity could be induced by wounding.

The expression of SWPA4 promoter by MV was also investigated. Particularly, leaf disks which were 7 mm in diameter were taken from a mature leaf. 20 disks were floated on each petridish containing 3 μM MV solution, which was cultured at 25℃ for 12 hours under darkness and then cultured under the light again. Upon completing

the culture, GUS activity was measured to investigate the expression of SWPA4 promoter by MV. As a result, the expression of the promoter was strongly induced in SWPA4 deletion promoter plants 12 hours after the light-culture. The expression of GUS in a transgenic plant infected with p1467 vector was 2.5 fold higher than that in CaMV 35S promoter. And also, the expressions of GUS in transgenic plants infected with p2433 and p433 were each 1.8 fold and 2.1 fold increased, comparing to that in CaMV 35S promoter (Table 1).

In addition, a transgenic tobacco plant, in which a deletion mutant of peroxidase genomic gene SWPA4 promoter was inserted, was infected with a pathogenic bacterium (*Pseudomonas syringae* cv. Tabaci) causing wild fire disease in tobacco plants. 48 hours later, the activity of induced GUS was measured. As a result, the expressions of GUS in transgenic plants infected with p2433, p1467 and p433, respectively, were 2.2 fold, 2.7 fold and 2.0 fold increased each, comparing to that in CaMV 35S promoter (Table 1). Thus, the activities of GUS in all the three transgenic plant cases were greatly increased, comparing to that in control.

<Table 1>

Changes of GUS activity in transgenic tobacco plants by wounding, treating methyl viologen (MV) and treating a pathogenic bacterium (*Pseudomonas syringae* cv. tabaci)

Expression vector	CaMV 35S	p2433	p1467	p433
Control	100%	8±2	12±3	6±1
Wounding	100%	65±5	250±10	102±8
MV	100%	180±11	315±22	210±11
P. syringae	100%	220±19	270±20	200±15

Taking all the above results into consideration, the activity of *SWPA4* promoter was confirmed to be highly induced by non-biological or biological stresses. Thus, *SWPA4* promoter of the present invention could be effectively used for the development of industrial multiple stress-resistant transgenic plants.

Example 6: Investigation of GUS expression in transgenic plant cells by using *SWPA4* promoter

<6-1> Preparation of transgenic culture cells

In order to produce transgenic culture cells, leaves of a transgenic tobacco plant in which a foreign gene was inserted were cultured in a callus-inducing medium prepared by adding 0.1 mg/ℓ BAP, 2 mg/ℓ NAA and 30 g/ℓ sucrose to MS basal medium and callus was induced thereon by a conventional procedure. Particularly, calli were induced using transgenic tobacco leaves each infected with transforming vectors p2433, p1934, p1467, p1199, p818, p433, p366, p306, p177 and p110. Among those calli, the one that was infected with p1467 (-1467 deletion promoter) vector showed the highest promoter activity, which was named 'p1467 (*Nicotiana tabacum cv. Xanthi*) cell line' and deposited at Korean Collection for Type Culture (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on Feb. 10, 2004 (Accession No: KCTC 10594BP).

<6-2> Investigation of a promoter activity in transgenic calli

In order to investigate how the expression of *SWPA4* promoter of the present invention could affect or regulate culture cell proliferation, GUS

activities in transgenic calli induced in transgenic plants infected with p2433, p1467, p433 and pBI121, respectively were measured. The measurement of GUS activity was performed following the same procedure above.

As a result, GUS activity (pmol/minute/mg protein) of plant culture cells containing *SWPA4* promoter was 12,000 ± 250, which was a percentage (%) to GUS activity of culture cells containing CaMV 35S promoter. All the GUS activities were higher in transgenic calli than in cells having CaMV 35S promoter, and especially, the callus infected with p1467 showed about 4.7 fold higher activity than that having CaMV 35S promoter. Calli, each infected with p2433 and p433, showed 3.1 fold and 2.5 fold higher activity respectively than a control (Table 2). Therefore, *SWPA4* promoter was proved to be effectively used for the development of an industrial transgenic culture cell line available for producing a high-value protein.

<Table 2>

GUS activity of transgenic tobacco culture cells (calli)

Expression vector	CaMV 35S	p2433	p1467	p433
GUS activity	100%	310±15%	470±25%	250±10%

INDUSTRIAL APPLICABILITY

As explained hereinbefore, a promoter of peroxidase genomic gene SWPA4 includes many regions especially recognizing lots of environmental stresses and has at least 8-fold higher promoter activity than CaMV 35S promoter which has been widely used to induce an expression of a target gene in a transgenic plant. Therefore, the use of a plant or plant culture cells, in which the promoter of the present invention is inserted, facilitates the development of an environmental stress-resistant plant and the development of a transgenic organism mass-producing valuable substances.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily

utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : KWAK, Sang-Soo
Korea Research Institute of Bioscience and Biotechnology (KRIBB),
#52, Oun-dong, Yusong-gu, Daejon 305-333,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
n1407 (*Nicotiana tabacum* cv. Xanthi) cell line	KCTC 10594BP

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on February 10 2004.

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): PARK, Yong-Ha, Director Date: February 23 2004

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